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Quest Summer Fellows, 2013

DETECTING THE SOURCE

Riboprobe Detection of APP mRNA to Delineate Aβ Protein Expression in a Rhesus Macaque Model of Age-Related Macular Degeneration (AMD)

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OVERVIEW

During my 2013 Quest Summer Fellowship I created molecular tools for detecting disease-associated gene expression at the mRNA level, termed "riboprobes." The riboprobes I have created are intended for application in the diseased Rhesus macaque retina, which serves as a powerful model of human age-related macular degeneration (AMD). In the following, I intend to provide a description of my research that is accessible to a general audience, yet informative about the nature and some details of the project. For additional information regarding experimental methods and results of this research, please refer to my final Keystone project.

BACKGROUND

Age-related Macular Degeneration (AMD) is the leading cause of vision loss in the elderly of industrialized countries (Johnson et al., 2003). Currently, no preventative cure exists for the early stages of AMD. In order to develop treatment, it is necessary to better understand how molecular and cellular events contribute to visual deterioration in people who suffer from this disease. In particular, the beta-amyloid (Aβ) protein, which has neurotoxic effects and has been studied extensively in the context of Alzheimer's disease (Cohen et al., 1998), may have an important role in the progression of AMD (Kam, Lenassi, & Jeffery, 2010; Yoshida et al., 2005). During my previous research at the Oregon National Primate Research Center (ONPRC) in the lab of Dr. Trevor McGill, we discovered an irregular pattern of Aβ protein expression in diseased Rhesus Macaque retinas, which are a powerful model of human AMD (Figure 1; Lee,

McGill, & Neuringer, 2012). Notably, this research involved the use of fluorescently labeled antibodies against the Aβ protein. This pattern of Aβ protein expression is irregular in that two separate antibodies that are both specific to the Aβ protein (4G8 and 6E10) identified expression in two mutually distinct layers of the retina, rather than in the same location (Figure 1). Over the course of my summer fellowship this year, I developed mRNA detection probes, or "riboprobes," to detect disease-associated gene expression in the Rhesus macaque model of human AMD. In particular, these riboprobes are intended to detect the expression of APP mRNA, which is a precursor to the Aβ protein. Thus, detecting the APP gene at the mRNA level using riboprobes may delineate the cause of the irregular Aβ protein expression seen at the ONPRC, and indicate which cells in the retina are the initial source of the Aβ protein. Subsequently, these APP riboprobes may further reveal how this type of gene expression, and subsequent Aβ accumulation is likely involved in the etiology of AMD.

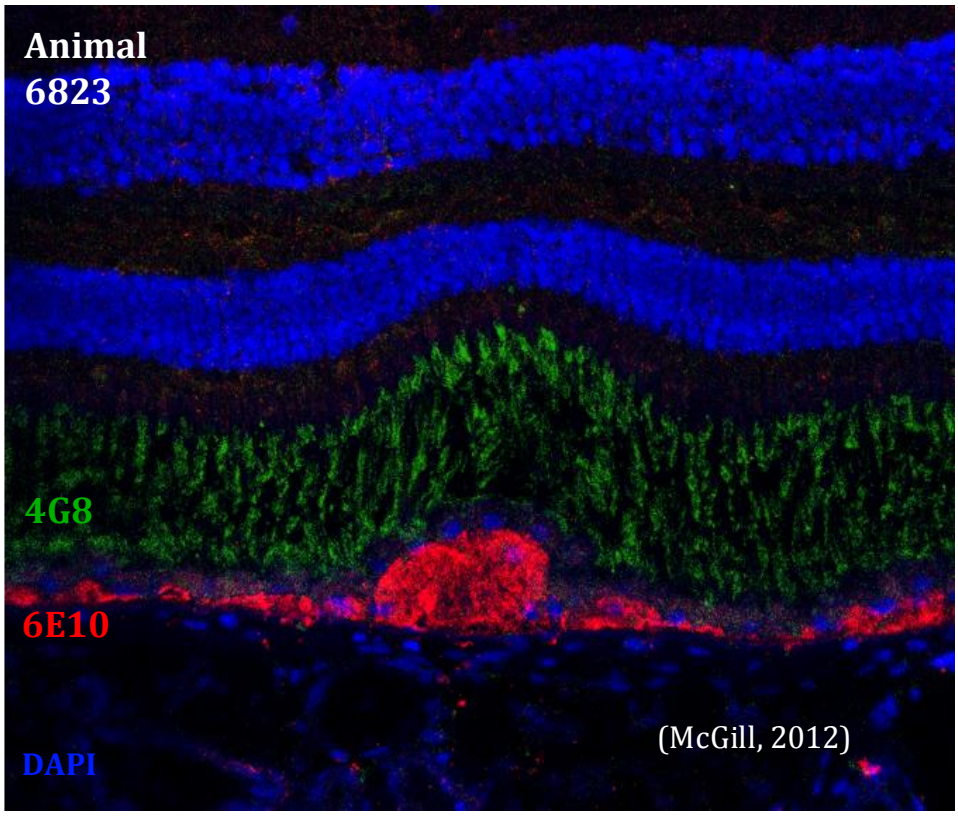

Figure 1. Two antibodies that bind to beta-Amyloid, 6E10 (1-16), and 4G8 (17-42) stain distinct loci of drusen-affected macaque retina. 6E10 (red) is immunoreactive in sub-RPE drusen deposits of the retina within Bruch's membrane, and 4G8 (green) is reactive in the outer segments in photoreceptors. DAPI was used as a nuclear marker to identify cell bodies (blue).

RIBOPROBES

The central dogma of molecular biology states that genetic information in DNA is transcribed into messenger RNA (mRNA), and mRNA is translated into protein (Figure 2-A). Proteins are the functional units of all cells. In diseased states, however, abnormal protein expression occurs, which causes cells to function irregularly and can lead to cell death. Additionally, after being made in cells proteins can be transported throughout different regions of tissue and the rest of the body. Therefore, detecting the location of proteins in tissue using antibodies is not a direct indication of where such proteins are sourced, due to their mobile nature in tissue. In order to determine the initial source of abnormal protein expression, it is necessary to detect the respective mRNA of the protein. Riboprobes are single-stranded sections of RNA that bind to mRNA expressed in tissue. Such probes are used to identify the location of mRNA prior to its translation into protein with the use of fluorescent labels or dyes (Figure 2-B). Therefore, when applied to diseased tissue, riboprobes are able to bind to mRNA of diseased proteins, and reveal the source of disease-causing gene and protein expression.

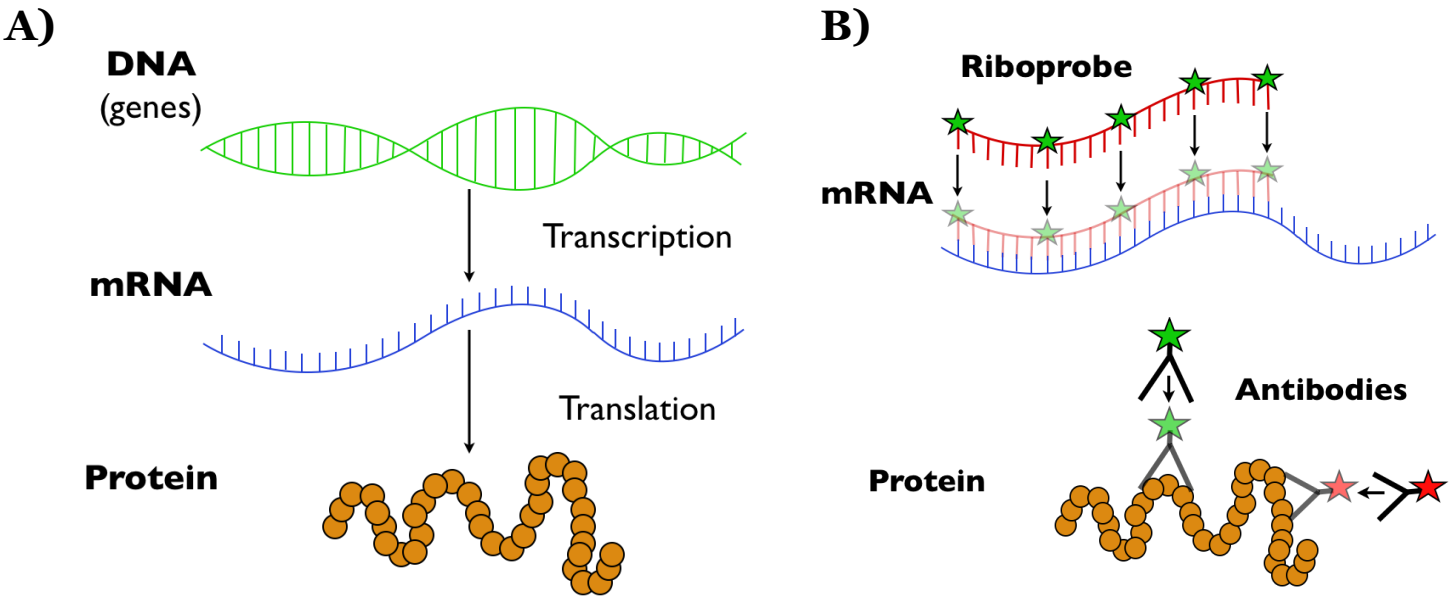

Figure 2. A) Schematic representing the central dogma of molecular biology, which states that DNA is transcribed into mRNA, which is then translated into protein. **B)** Representation of how riboprobes bind to mRNA, and antibodies bind to protein. Both riboprobes and antibodies have fluorescent markers added for their subsequent visualization in tissue.

RIBOPROBE SYNTHESIS

Creating riboprobes is an intricate process that involves gathering information about the mRNA sequence of interest through online databases, which is followed by a careful design process and various laboratory experiments. I will explain the process of creating riboprobes in the context of the riboprobe that I have made to detect APP mRNA expression in the Rhesus macaque retina[1].

1) BIOINFORMATICS

Designing riboprobes begins with collecting information about the mRNA sequence that the riboprobe is intended to detect. mRNA sequences are stored in online databases as complementary DNA (cDNA) sequences for various organisms. The cDNA sequence that represents Rhesus Macaque APP mRNA is 3452 nucleotides in length, and a particular portion of this sequence will be used for subsequent analysis, riboprobe design, and synthesis.
To view this sequence in full, use the following link:
http://www.ncbi.nlm.nih.gov/nuccore/XM_002803170.1

2) PRIMER DESIGN

Primers are short (15-25 nucleotide) single-stranded DNA oligomers that bind to sections DNA or RNA in a genetic sample. Typically, two primers are designed to bind to sections of a DNA or RNA that flank a gene of interest (Figure 3). With the addition of various reagents that include enzymes and salt solutions, these primers allow enzymes to create copies of the gene of interest from a DNA or RNA sample. The specific copy of the APP mRNA sequence will also determine the subsequent riboprobe synthesized from those copies following manipulation. Thus, it is necessary to design primers that will allow for a riboprobe that meets desired parameters for application. Such parameters may include size requirements, and regions of interest for detection in the APP mRNA sequence.

[1] I have also created other versions of the APP mRNA riboprobe, in addition to a positive control probe against Rhesus Parvalbumin mRNA, which is widely expressed in the retina. For simplicity, I will not be elaborate on these additional riboprobes here. Nonetheless, the synthesis process is essentially identical for these other probes. If interested, please refer to my Keystone project for information regarding these other riboprobes I have made.

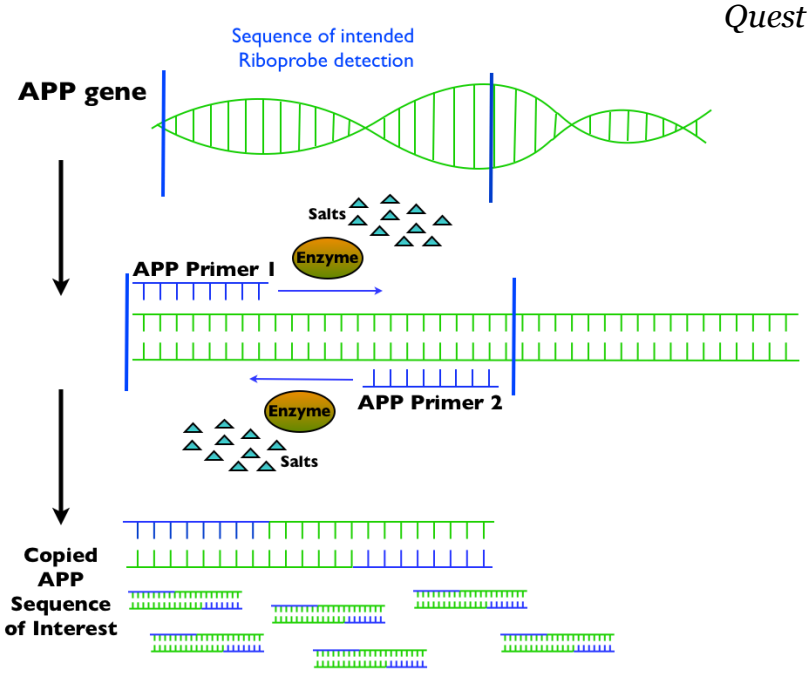

Figure 3. Diagram generally illustrates how primers create copies of a specific region of a genetic sequence. A primer set (APP Primers 1 and 2) flanks a gene of interest, and amplifies the specific region with the addition of enzymes and salts.

I designed primers that make DNA and RNA copies of a particular section of the Rhesus macaque APP mRNA sequence, which is necessary to create riboprobes. Notably, mRNA can be altered into different isoforms following transcription. APP mRNA is altered into three isoforms within a particular region of the total sequence. Consequently, I carefully designed primers to make copies of a section of the APP mRNA sequence that is not altered or modified when expressed in retinal tissue. Therefore, my riboprobe detects all different isoforms of Rhesus macaque APP mRNA in the retina (Figure 4).

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Figure 4. Schematic shows a region of transcriptional modification in APP mRNA, and how my riboprobe detects an mRNA region that is not modified.

Due to the specific length of the section of APP mRNA that my primers create copies of, my riboprobe is 373 nucleotides in length. This cDNA sequence of my riboprobe, along with the respective primer binding sites, is shown below:

5'- TGGCACTGCTCCTGCTGGCCGCCTGGACGGCTCGGGCGCTGGAGGTACCTACTGATGGCAAT
GCTGGCCTGCTGGCTGAACCCCAGATCGCCATGTTCTGTGGCAGACTGAACATGCACATGAATG
TCCAGAATGGGAAGTGGGATTCAGATCCATCAGGGACCAAAACCTGCATTGATACCAAGGAAGG
CATCCTGCAGTATTGCCAGGAAGTCTACCCTGAACTGCAGATCACCAATGTGGTAGAAGCCAACC
AACCAGTGACCATCCAGAACTGGTGCAAGCGGGGCCGCAAGCAGTGCAAGACCCATCCCCACTT
TGTGATTCCCTACCGCTGCTTAGTTGGTGAGTTTGTAAGCGATGCCCTTCTCGT - 3'
(Primer sites highlighted in blue)

3) REVERSE TRANSCRIPTION

Transcription is the process of DNA being transcribed into RNA. Therefore, reverse transcription is the process of making cDNA from RNA, which is the reverse of typical transcription that occurs *in-vivo*. DNA is less susceptible to degradation over time than RNA due to differences in chemical structure. Thus, making cDNA from RNA is necessary for experimentation with any genetic sequence of interest. Using various enzymes, I created cDNA from RNA isolated from Rhesus macaque brain tissue at the Oregon National Primate Research Center (ONPRC), which was provided as a generous gift from Dr. Trevor McGill and Dr. Jonathan Stoddard. This allows for subsequent manipulation of the APP mRNA sequence in a DNA form, which is required for riboprobe synthesis.

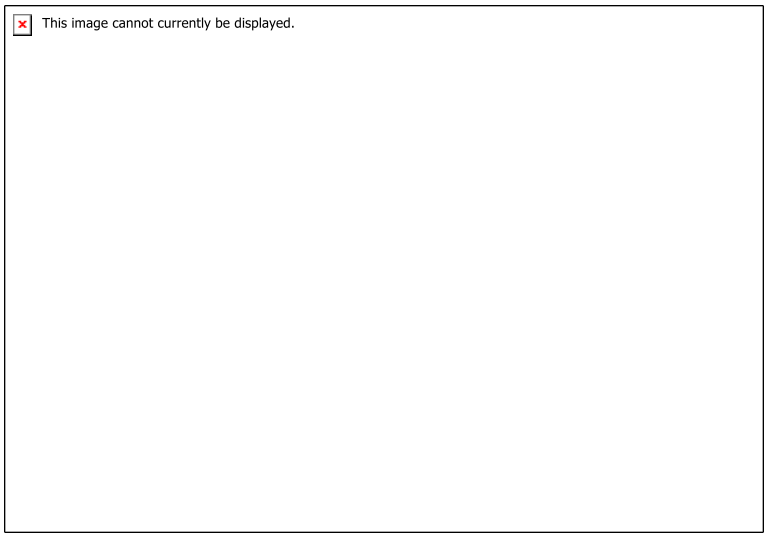


Figure 5. Diagram illustrating the general process of reverse transcription, in which RNA isolated from tissue is reverse transcribed into cDNA.

4) PCR

Once I created cDNA from Rhesus macaque brain RNA, I then made copies of the Rhesus macaque APP sequence using a technique called the polymerase chain reaction (PCR). PCR is a fundamental technique in molecular biology used to amplify and alter genetic sequences. Following the initial amplification of the APP sequence using PCR (Figure 6-A; PCR#1), I was also able to insert promoter sequences into the APP gene using a second round of PCR (Figure 6-B; PCR#2). Promoter sequences act as "start sites" to create riboprobes from the amplified APP sequence. Moreover, based on the side of the APP gene in which I insert these promoter sequences, I am able to make non-complementary (sense) and complementary (antisense) riboprobes to APP mRNA.

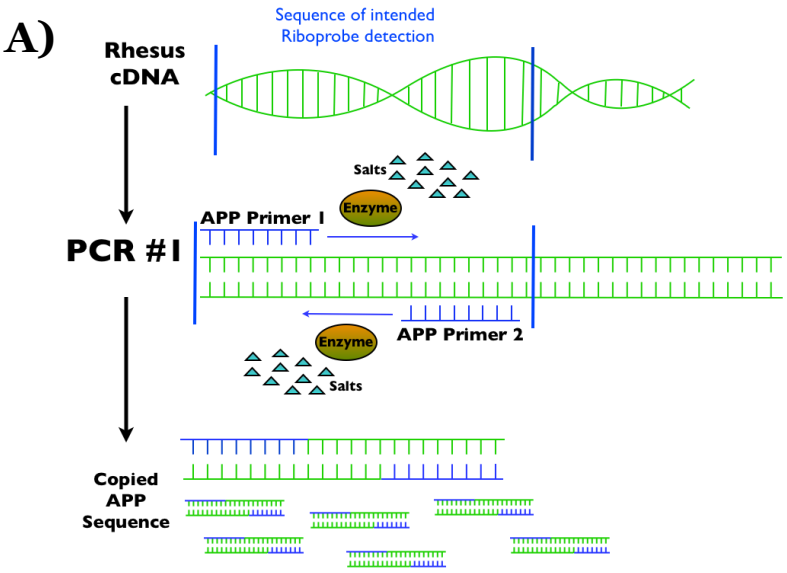
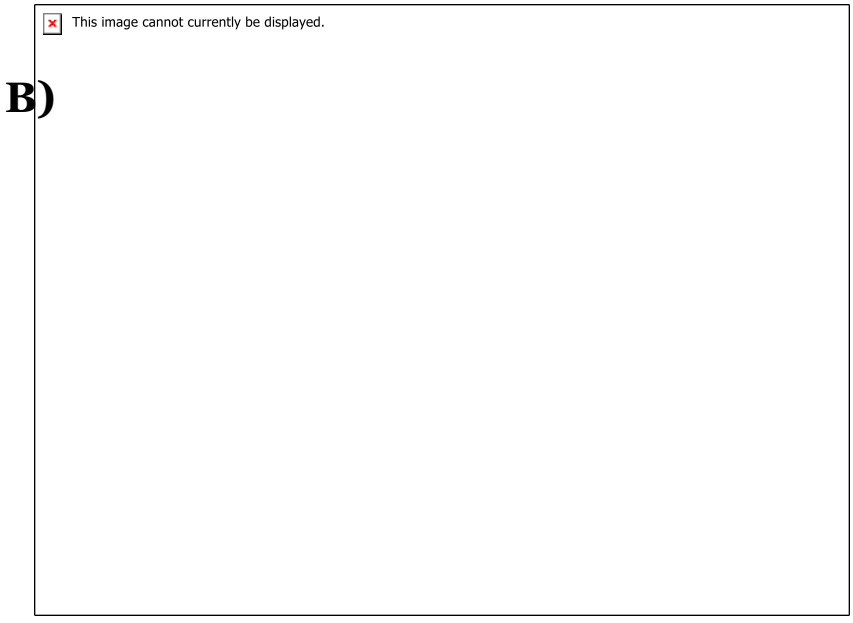


Figure 6. A) Schematic illustrating the first round of PCR, which copies a specific section of the APP gene from cDNA. **B)** A second round of PCR using APP primers tailed with promoter sequences causes the incorporation of promoter regions into the copied APP sequence for subsequent riboprobe synthesis.



5) IN-VITRO TRANSCRIPTION

In-vitro transcription involves creating RNA strands from DNA products following PCR. Enzymes termed RNA polymerases synthesize RNA strands from PCR products starting at promoter sequences. Promoter sequences signal to RNA polymerases to "start here" for creating strands of RNA. The particular system used in my experimental design is called the T7-T3 in-vitro transcription system. This method allows me to select which strand of RNA, either sense (T7) or antisense (T3) to use to create a riboprobe. mRNA expressed in tissue always represents the sense strand of a genetic sequence. Therefore, antisense riboprobes, which in my system are transcribed from the T3 promoter sequence, will bind to and detect mRNA expressed in tissue (Figure 7). In addition, sense riboprobes will represent the same genetic sequence as mRNA expressed in tissue, and will subsequently not bind to mRNA. Thus, the antisense (T3) riboprobe will detect APP mRNA, whereas the sense (T7) riboprobe will not detect APP mRNA, serving as a negative control probe. In addition, adding labeled ribonucleotides causes the incorporation of fluorescent tags into the riboprobe (Figure 7). This allows for visualization of the riboprobe, and subsequently, the presence and location of APP mRNA in tissue.

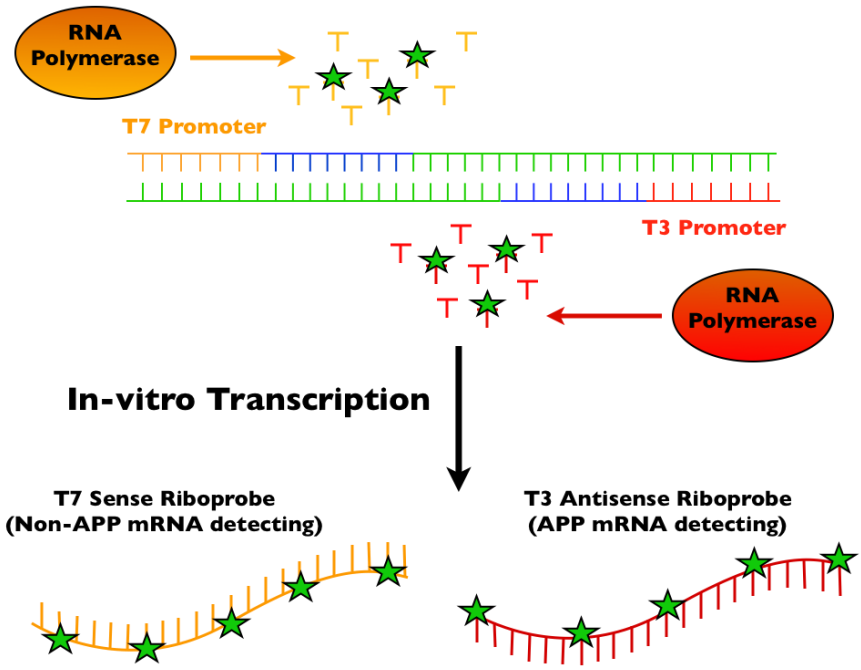


Figure 7. During in-vitro transcription RNA polymerases synthesize single strands of RNA from respective promoter sites, which act as "start signals" for transcription. The addition of a proportion of fluorescently labeled ribonucleotides causes the incorporation of fluorescent labels into each riboprobe.

6) GEL ELECTROPHORESIS

Following PCR and In-vitro Transcription, it is necessary to confirm that the correct products have been successfully synthesized during the experiment. The presence of PCR (DNA) or In-vitro Transcription (RNA) product is examined using a technique called gel electrophoresis. In this technique, a small amount of DNA or RNA product is removed from a sample, and placed into wells formed in a porous gel material. The gel is immersed in a saline solution, and electrodes are placed at either end of the gel. Both RNA and DNA possess a negative charge, and thus, when electric current is run through the saline solution in which the gel is immersed, the RNA or DNA products are forced through the porous gel material. Given that RNA and DNA products have specific sequence lengths, the products will separate based on their sizes. Following incubation in ethidium bromide, which stains the RNA and DNA products, the gel is imaged under a UV light, which causes the products to fluoresce in the gel. If the correct DNA or RNA product has been made, a fluorescent band will form within a specific region of the gel that corresponds to the appropriate size of the sequence. Given that the amplified APP DNA sequence, along with the subsequent riboprobe is 373 nucleotides in length, we should expect to see a band in this size range. As shown in the gel images below, I have successfully produced the APP PCR products (Figure 8), along with the pair of Rhesus macaque APP riboprobes (Figure 9).

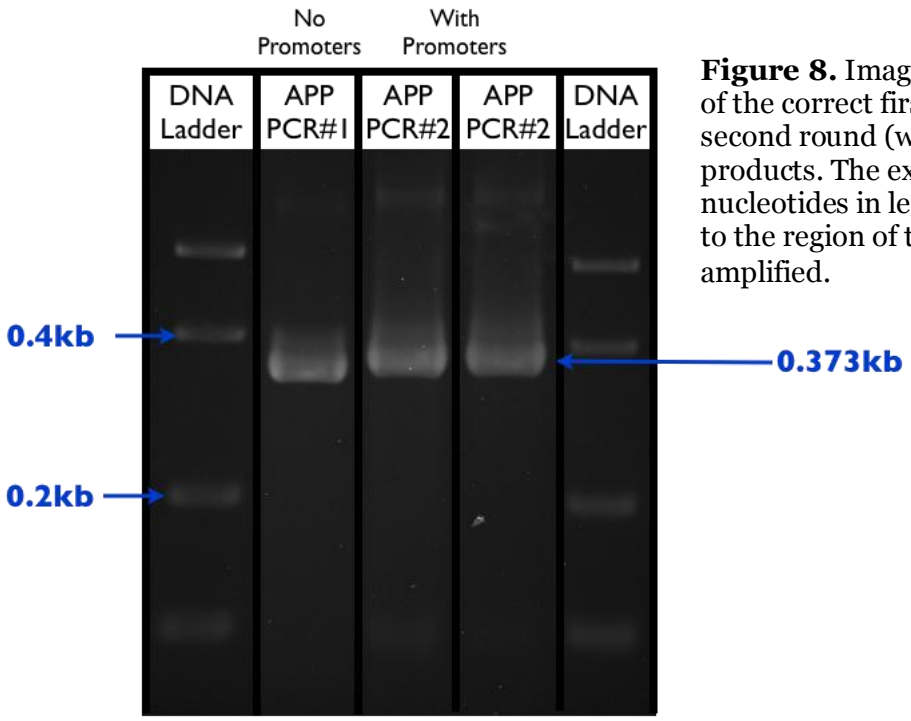


Figure 8. Image indicates the presence of the correct first (no promoters) and second round (with promoters) PCR products. The expected product is 373 nucleotides in length, which corresponds to the region of the APP sequence being amplified.

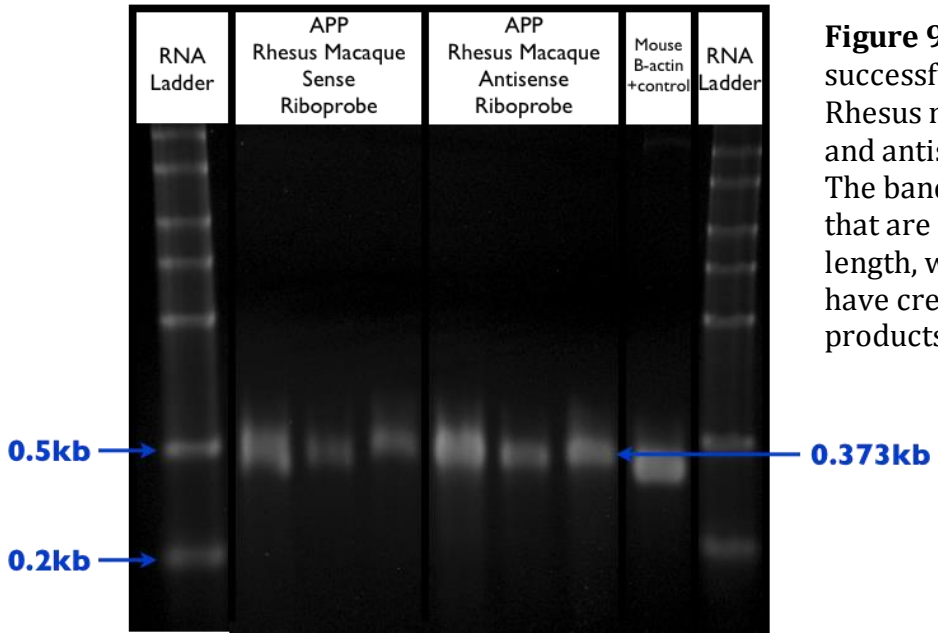


Figure 9. Image shows the successful synthesis of Rhesus macaque APP sense and antisense riboprobes. The bands indicate products that are 373 nucleotides in length, which confirms that I have created the intended products.

IN-SITU HYBRIDIZATION

Once the successful synthesis of the riboprobe has been confirmed with gel electrophoresis, the final stage of testing is the application of the riboprobe in tissue. In-situ hybridization is the technique used to apply riboprobes to tissue to detect disease-associated mRNA expression. In this technique, a solution containing the riboprobe is washed over a thin section of tissue, and allowed to incubate for a period of time. If the mRNA is expressed in the tissue that the riboprobe is applied against, then the riboprobe will bind to the correct mRNA within cells that express the transcript. However, if the mRNA is not expressed in tissue that the riboprobe is applied to, then the riboprobe will not reveal the expression of the appropriate transcript. This stage of the experiment will be performed in the upcoming months at the Oregon National Primate Research Center (ONPRC) on sections of Rhesus macaque retinal tissue. Based on previous evidence of APP expression in other studies, and in corroboration with the regions in which we have identified expression of the Aβ protein, APP mRNA will most likely be expressed in retinal pigmented epithelial (RPE) cells in the Rhesus macaque retina. The irregular Aβ protein expression that we have identified with

antibodies in the Rhesus macaque retina is localized in Bruch's membrane and the outer segments of photoreceptors. It may be that APP mRNA expression, which results in expression of the Aβ protein, is located between Bruch's membrane and photoreceptor outer segments.

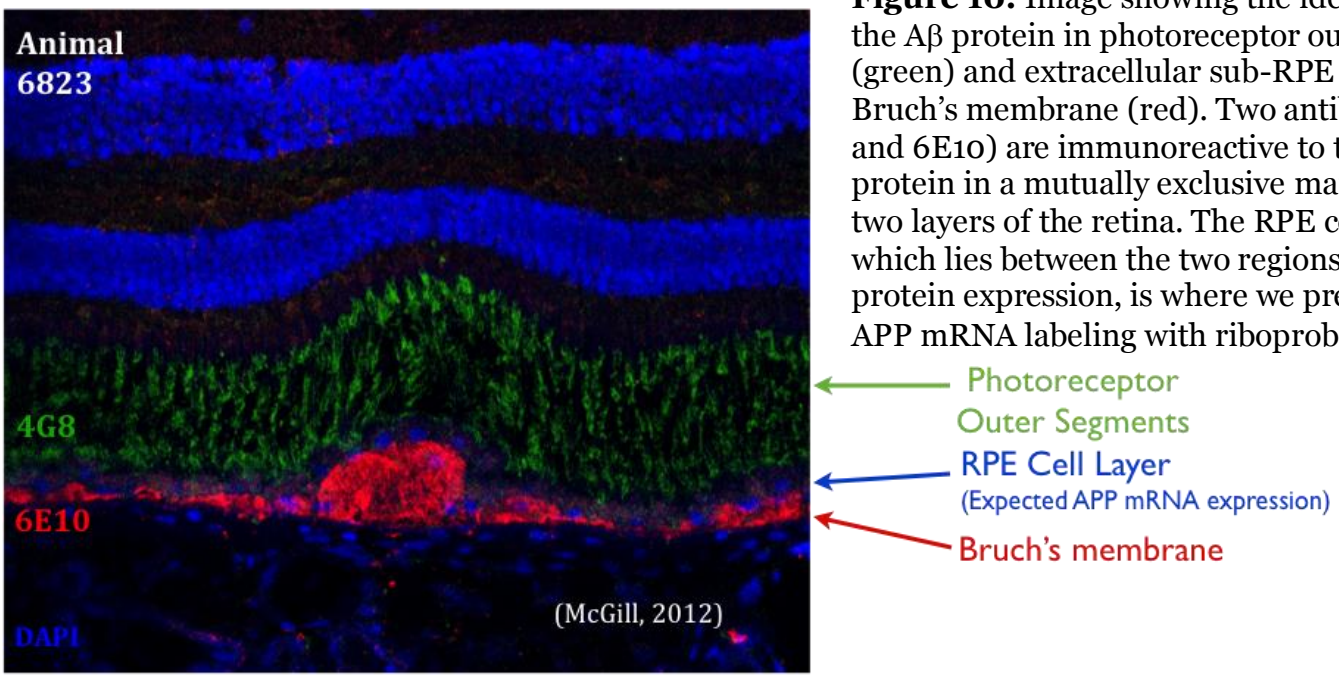


Figure 10. Image showing the identification of the Aβ protein in photoreceptor outer segments (green) and extracellular sub-RPE deposits in Bruch's membrane (red). Two antibodies (4G8 and 6E10) are immunoreactive to the Aβ protein in a mutually exclusive manner in the two layers of the retina. The RPE cell layer, which lies between the two regions of Aβ protein expression, is where we predict to find APP mRNA labeling with riboprobes.

DISCUSSION

Over the course of my 2013 Summer Fellowship I successfully designed and synthesized riboprobes to detect Rhesus macaque APP mRNA. Currently, my riboprobes are being applied to diseased Rhesus macaque retinal tissue in order to determine the presence of APP mRNA at the ONPRC. Through this process, our group aims to identify the source of expression of the Aβ protein in the retina, which is likely involved in visual deterioration in AMD (Anderson et al., 2004; Yoshida et al., 2005). Our results will help elucidate how APP mRNA, and subsequent Aβ protein expression may be involved in the etiology of this disease. Thus far, we have identified Aβ protein expression in both photoreceptor outer segments and in extracellular deposits above Bruch's membrane (Figure 1; Figure 10; Lee, McGill, & Neuringer, 2012). Although groups have reported these two findings separately in aged human patients (Dentchev et al., 2003; Seth et al.,

2008), and rodent models (Kam, Lenassi, & Jeffery, 2010; Yoshida et al., 2005), the two patterns of Aβ protein expression have yet to be reported concomitantly. The pattern of Aβ protein staining we have identified may indicate that APP mRNA expression occurs within the RPE cell layer, which is between the two regions of the Aβ protein accumulation (Figure 10). Studies from Yoshida et al. (2005), and Seth et al. (2008) have identified APP expression in the RPE layer of rodent models of human AMD, and in aged human donor eyes, respectively. Moreover, various groups have hypothesized that RPE cells have an important role in the progression of vision loss in AMD through the expression of the APP protein, the Aβ protein, and factors associated with immune response (Johnson et al., 2011; Kam, Lenassi, & Jeffery, 2010; Shimazawa et al., 2008). Similarly, the expression of these factors is a hallmark of Alzheimer's disease (AD), and their involvement in cell loss in the brain and age-related cognitive decline is well characterized (Cohen et al., 1998; Hardy, & Selkoe, 2002). Studies using amyloid and immune-targeted therapies often applied in the context of AD have been successful in ameliorating the progression of vision loss in rodent models of AMD (Ding et al., 2008; Chi, Yoshida, Lambris, & Iwata, 2010). Thus, elucidating how these factors may be involved in the progression of AMD will also indicate how this disease is similar to other age-related pathologies, and how therapies can be applied across such conditions.

It is also possible that we may not identify APP mRNA expression in the retinas of Rhesus macaques located at the ONPRC. Previously, our group was unable to identify APP protein expression in this model of human AMD (Data not shown). One factor that could influence these data is the diet of Rhesus macaques at the ONPRC, which is considered much healthier than that of human subjects who show APP protein expression in the retina. Indeed, previous studies have supported that diet is a strong determining risk factor for human patients who develop AMD (Anand et al., 2000; Cho et al., 2004). However, a lack of APP protein expression implies that the source of Aβ protein expression may not be within the retina in Rhesus macaques. Nonetheless, antibodies used to detect the APP protein are not likely to be as sensitive to detection as the riboprobes

described here. Thus, it is also possible that we may identify APP mRNA in the absence of APP protein as identified with antibodies. In such a case, our results will illustrate the importance of a multiple-methods approach in analyzing histological evidence regarding the etiology of AMD. Furthermore, the use of APP riboprobes will aid in further characterizing and validating the Rhesus macaque as a powerful model of human AMD. Overall, this research aims to help develop targeted therapies that are not currently available for people suffering from this condition.

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